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Short Communication

An on-line, column-switching high-performance liquid chromatographic procedure for the removal of probenecid from human plasma, serum, or urine in the quantitative determination of cefmetazole or cefoxitin

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Introduction

Probenecid is commonly used for the inhibition of renal tubular secretion and is useful in prolonging inhibitory concentrations of those antibiotics which are primarily eliminated via renal tubular secretion. Cefmetazole sodium is a semisynthetic derivative of cephamycin C [1], which has broad-spectrum gram-positive and gram-negative antibacterial activity. Cefoxitin is a second-generation cephalosporin derivative of cephamycin with good activity against gram-negative organisms. Both cefmetazole sodium and cefoxitin are eliminated in man primarily via renal tubular secretion. Thus their half-lives would be expected to be prolonged through the preadministration of probenecid. Because of the widespread use of probenecid, a procedure to remove probenecid from clinical biomatrix specimens to allow quantitation of the desired antibiotic, would appear to have wide application in the bioanalytical field. Column-switching techniques have been used in this laboratory to separate analytes of interest from endogenous substances [2]. Because of the vast differences in polarity between probenecid and the cephalosporins, cefmetazole and cefoxitin, solid-phase extraction (SPE) could have been used to remove probenecid selectively, prior to HPLC analysis. However, in this laboratory, on-line column-switching techniques have proved to be less labour intensive and not subject

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to the variability of SPE cartridges. Sekine *et al.* [3] have described an HPLC method for the determination of cefmetazole in serum which utilized deproteinization with 5% trichloroacetic acid (TCA) in methanol and a mobile phase of citrate buffer (pH 5.4; 5 mM)–acetonitrile (85:15, v/v). However, work in this laboratory has revealed that the high concentration of TCA that Sekine *et al.* [3] used produced chromatographic artifacts and prevented volumes $>\sim 10$ µl from being injected. In order to automate the chromatography and to achieve the maximum sensitivity, it was necessary to introduce a larger fraction of the sample into the HPLC. Consequently a modified precipitation procedure was required to avoid exceeding the buffering capacity of the mobile phase. The coupling of an on-line column-switching technique for the removal of probenecid with the improved deproteinization procedure resulted in a method which was applicable to the analysis of selected cephamycins and cephalosporins in biological fluids.

Experimental

Reagents

All solvents were UV grade and distilled in glass (Burdick and Jackson Laboratories, Muskegon, MI, USA). Sodium citrate, citric acid and trichloroacetic acid were AR grade (Mallinkrodt, Paris, KY, USA). Water was Milli-Q (Millipore, Milford, MA, USA) or equivalent purity.

Internal standard-protein precipitation solutions

Barbital (Fisher Scientific, Pittsburgh, PA, USA) was used as the internal standard for both analytes in all three matrices. Serum and plasma were spiked with a solution containing the internal standard at a concentration of about 1.5 mg ml^{-1} in methanol–TCA (99.5:0.5, v/v) and the urine was spiked with a 3.0-mg ml^{-1} solution of barbital in the same solvent.

Apparatus

The chromatographic system consisted of a Kratos Analytical Spectroflow 400 pump for the analytical separation, and a Beckman Model 110A pump for the on-line clean-up. The HPLC detector was a Kratos Analytical Spectroflow Model 783, fitted with an 8-µl cell and operated at 254 nm. The detector output was monitored with a Linear Model 585 chart recorder and a Harris 1000 computer system. A Hitachi Model 655A-40 autosampler introduced the sample into the chromatographic system. The analytical separation was performed on a Spherisorb ODS precolumn (5 µm, 10 × 4.6 mm; Alltech Associates, Deerfield, IL, USA) and a Zorbax C18 column (7 µm, 250 × 4.6 mm; Alltech Associates, Deerfield, IL, USA) or a Supelcosil LC18 column (5 µm, 250 × 4.6 mm; Supelco, Bellefonte, PA, USA). The clean-up was performed on a Brownlee OD-GU RP-18 guard column (5 µm, 30 × 4.6 mm). The column-switching device consisted of a Valco CV-6-HPax valve equipped with a pneumatic actuator. The valve was actuated by a two-stage timing device manufactured in this laboratory and triggered by the autosampler upon sample injection. A µBondapak C18 column (10 µm, 300 × 3.9 mm) was connected to the waste line of the clean-up system to provide sufficient resistance to minimize pressure fluctuations during the column-switching procedure. The mobile phase for the elution of both systems was acetonitrile/sodium citrate–citric acid buffer (pH 5.4; 0.01 M; 12:18, v/v). The flow rates were 1.5 ml min^{-1} on the clean-up side and 2.0 ml min^{-1} on the analytical side.

Serum or plasma calibration standards

Calibration standards of either cefmetazole or cefoxitin were prepared by accurately weighing approximately 20.0 mg of reference standard material (cefmetazole free acid, Sankyo Company Ltd, Tokyo, Japan, or cefoxitin sodium salt, Merck, Sharpe & Dohme, West Point, PA, USA) into 10-ml volumetric flasks and dissolving with water. Using volumetric glassware, 1:1 serial dilutions with water were made to achieve standard concentrations of 1, 2, 4, 8, 16, 32, 64, 125, 250, 500, 1000 and 2000 μg ml^{-1}.

Urine calibration standards

Calibration curve standards for cefmetazole were prepared by accurately weighing approximately 100 mg of the reference standard material (cefmetazole) into a 10-ml volumetric flask and dissolving and diluting with sodium citrate–citric acid buffer (pH 5.4; 0.2 M). Using volumetric glassware, 1:1 serial dilutions were made with citrate buffer to achieve concentrations of 0.15, 0.30, 0.60, 1.2, 2.5, 5.0 and 10.0 mg ml^{-1}. Calibration standards for cefoxitin sodium salt were prepared in a similar fashion, except that water was used as the diluent.

Spiked serum and plasma controls

Spiked controls were prepared by accurately weighing either cefmetazole or cefoxitin sodium salt reference standard material into volumetric flasks, and dissolving and diluting with normal human plasma or serum to achieve final concentrations of approximately 5, 25 and 125 μg ml^{-1}.

Spiked urine controls

Spiked controls were prepared by accurately weighing either cefmetazole or cefoxitin reference standard material into volumetric flasks and dissolving and diluting with normal human urine to achieve final concentrations of about 60, 240 and 600 μg ml^{-1}.

Sample preparation

An aliquot (100 μl) of each standard solution for either cefmetazole or cefoxitin was added to 1.0 ml of normal human serum, plasma or urine. An aliquot (100 μl) of water was added to each sample tube containing 1.0 ml of spiked serum or plasma controls or subject specimens. For urine cefoxitin determinations, 100 μl of water was added to each 1.0 ml control or subject specimen, whilst for urine cefmetazole determinations, 100 μl of sodium citrate–citric acid buffer (pH 5.4; 0.2 M) was added to each control or subject specimen. All tubes were vortexed vigorously for 10 s to mix. Then 2.0 ml of the appropriate internal standard solution was added to each tube; the tubes were vortexed for 1 min, and centrifuged for 10 min at 600 ***g***. A measured aliquot of the supernatant was removed from each tube and mixed with an equal volume of sodium citrate–citric acid buffer (pH 5.4; 0.2 M) in the autosampler injection vial and vortexed to mix.

On-line sample clean-up and chromatography

A 50-μl aliquot of the buffered supernatant was introduced into the chromatograph with the Hitachi autosampler. The sample and mobile phase entered the clean-up side of the column-switching device with the Brownlee guard column in the "loop" position of the Valco valve. For the first 1.5 min, the clean-up column eluent entered the analytical column, this being sufficient time for cefmetazole, cefoxitin and the internal standard to elute from the clean-up column. The valve was then rotated and the direction of flow of

mobile phase on the clean-up guard column reversed for 10 min to remove any probenecid or other strongly retained components to waste. During this wash period, the mobile phase delivered by a second pump completed the separation of cefmetazole, cefoxitin and the internal standard on the analytical column. At the end of the wash period the valve was returned to its original position to re-equilibrate the precolumn with the mobile phase flowing in the forward direction for about 3.5 min prior to the next injection.

Results

Method development and assay validation

Various concentrations of TCA in methanol were tested to determine the optimum proportion for maximal drug recovery. In serum this was 0.5–1.0%, v/v, TCA in methanol for both cefmetazole and the internal standard. The internal standard had slightly better recovery from 1.0%, v/v, whilst cefmetazole had better recovery from 0.5%, v/v, TCA. Thus 0.5%, v/v, was chosen as the most appropriate concentration of TCA for protein precipitation.

Figure 1 shows overlaid chromatograms of cefmetazole and cefoxitin in representative pre-dose and post-dose serum samples. It can be seen that both the cefmetazole and the cefoxitin peaks are well resolved from the internal standard. Cefmetazole and cefoxitin did not exactly coelute, there being a small difference of about 0.2 min in their retention times. The limits of quantitation (LOQ) for cefoxitin were slightly better than those for cefmetazole, due to a minor interfering component that was better resolved from the cefoxitin peak than from the cefmetazole peak.

Figure 2 is a chromatogram from representative pre-dose and post-dose urines for cefmetazole and cefoxitin. As with chromatograms of the serum, cefoxitin and cefmetazole were well resolved from the internal standard and other endogenous components. In comparing Figs 1 and 2 a slight shift in the retention time of the cefmetazole and cefoxitin peaks between matrices was seen; this shift in retention time

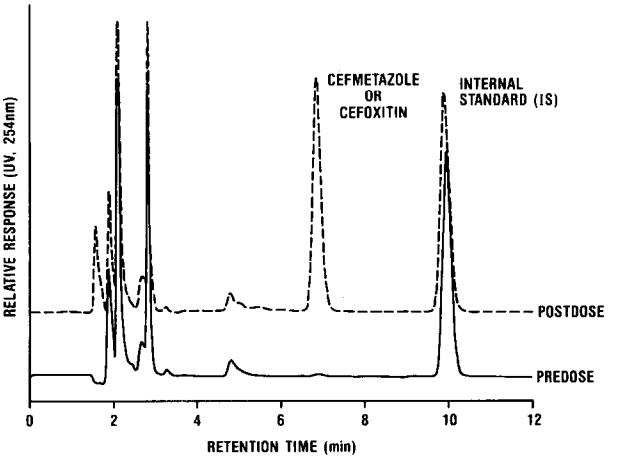

Figure 1
Representative chromatogram of cefmetazole or cefoxitin and the internal standard in human serum.

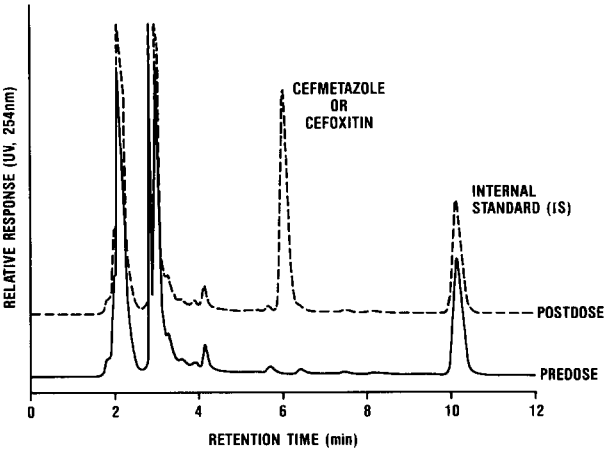

Figure 2
Representative chromatogram of cefmetazole or cefoxitin and the internal standard in human urine.

was attributed to the variation in the properties of the two chromatography columns used in these experiments.

Confirmation that probenecid was successfully removed from the analytical system was achieved by the analysis of two cefmetazole standard curves in serum, one containing 80 μg ml^{-1} of probenecid (corresponding to the approximate therapeutic level), and the other containing no probenecid. Unweighted linear regression analysis of the standards containing probenecid versus those without probenecid yielded a slope of 0.9915 ± 0.01056, an intercept insignificant from zero and a correlation coefficient of 0.9995. These data indicate that the probenecid was successfully removed by the on-line clean-up procedure.

A similar experiment was conducted to validate both serum and plasma matrices. Two cefmetazole standard curves were prepared, one in normal human serum, the other in normal, heparinized human plasma. Unweighted linear regression analysis of the standards in serum *versus* those in plasma yielded a slope of 0.9981 ± 0.005114, an intercept insignificant from zero, and a correlation coefficient of 0.9999. These data confirmed that there was no difference in the recoveries of cefmetazole from either human serum or human plasma.

Linearity

Serum. The linearity of the method was established by analysis of 17 cefmetazole and cefoxitin calibration curves each ranging from 2 to 200 μg ml^{-1}. In each case, both cefmetazole and cefoxitin intercepts were not significantly different from zero ($P > 0.05$) and all subsequent calibration curves were forced through the origin. The cefmetazole calibration curve correlation coefficients ranged from 0.9955 to 1.0. The within-day precision, expressed by the relative standard deviation (RSD) of the slope (forced through the origin) ranged from 0.2 to 2.6%. The between-day RSD of the slope (Table 1) was 11.2% for the cefmetazole calibration standards. The cefoxitin calibration curve correlation coefficients ranged from 0.9994 to 1.0, and the within-day RSD of the slope ranged from 0.3 to 0.8%. The between-day RSD of the slope (Table 1) was 10.7% for the cefoxitin calibration standards.

Table 1
Linearity and precision data for the analysis of cefmetazole and cefoxitin in serum

Concentration ($\mu g\ ml^{-1}$)	PHR*	SD	RSD (%)
Cefmetazole (n = 17)			
2.04	0.0339	0.0163	48.1
5.67	0.0710	0.0119	16.7
9.45	0.110	0.0182	16.6
15.8	0.166	0.0352	21.2
43.8	0.496	0.0585	11.8
72.9	0.932	0.0910	9.8
122	1.56	0.186	11.9
203	2.66	0.321	12.1
Slope	0.0130	0.00146	11.2
Correlation coefficient	0.9989	0.0014	1.4
Cefoxitin (n = 16)			
2.00	0.0297	0.00346	11.6
5.93	0.0857	0.00910	10.8
9.23	0.138	0.0148	10.7
15.4	0.0229	0.0251	10.9
42.7	0.633	0.0679	10.7
71.2	1.05	0.116	11.1
119	1.74	0.193	11.1
198	2.82	0.298	10.6
Slope	0.0144	0.00154	10.7
Correlation coefficient	0.9998	0.0001	0.01

*Mean peak height ratio.

Urine. Linearity for the method was established by analysis of nine cefmetazole and cefoxitin calibration curves ranging from 15 $\mu g\ ml^{-1}$ to 1 $mg\ ml^{-1}$. In each case, neither the cefmetazole nor the cefoxitin intercepts were significantly different from zero ($P > 0.05$), thus the slope calculated through the origin was used. The cefmetazole calibration curve correlation coefficients ranged from 0.9989 to 0.9998, and the within-day RSD of the slope ranged from 0.6 to 1.7%. The between-day RSD of the slope (Table 2) was 6.0% for the cefmetazole calibration standards. The cefoxitin calibration curve correlation coefficients ranged from 0.9985 to 0.9998, and the within-day RSD of the slope ranged from 0.8 to 1.9%. The between-day RSD of the slope (Table 2) was 6.5% for the cefoxitin calibration standards.

Precision

Serum. The precision of the method was measured by determining the RSD between runs for the peak height ratios of the cefmetazole (n = 17) and cefoxitin (n = 16) calibration curve standards, with respect to the internal standard. The RSD values for cefmetazole and cefoxitin are given in Table 1.

The LOD was calculated by estimation of s_0 [4] from the calibration curve standards for both cefmetazole and cefoxitin. The LOD was set at $3s_0$ as recommended by Keith *et al.* [4]. Using this method, the LOD for cefmetazole and cefoxitin was calculated to be 1.3 and 0.2 $\mu g\ ml^{-1}$, respectively (corresponding to 10.5 and 1.6 ng on column). The

Table 2
Linearity and precision data for the analysis of cefmetazole and cefoxitin in urine

Concentration (mg ml^{-1})	PHR*	SD	RSD (%)
Cefmetazole (n = 9)			
0.016	0.154	0.0119	7.8
0.031	0.299	0.0182	6.1
0.063	0.581	0.0348	6.0
0.13	1.15	0.0549	4.8
0.25	2.27	0.115	5.1
0.50	4.36	0.229	5.3
1.0	8.31	0.543	6.5
Slope	8.40	0.502	6.0
Correlation coefficient	0.9994	0.0003	0.03
Cefoxitin (n = 9)			
0.016	0.147	0.0135	9.2
0.031	0.278	0.0141	5.1
0.063	0.568	0.0180	3.2
0.13	1.14	0.0504	4.4
0.25	2.23	0.106	4.8
0.50	4.29	0.254	5.9
1.0	7.94	0.515	6.5
Slope	8.815	0.529	6.5
Correlation coefficient	0.9991	0.0004	0.04

*Mean peak height ratio.

LOQ was determined by estimating the concentration at which a between-day RSD of 25% would be obtained. Using the spiked control precision data, the LOQ for cefmetazole was estimated to be approximately 2 μg ml^{-1}, and 1 μg ml^{-1} for cefoxitin.

Urine. The precision of the method was determined in a similar fashion to that for serum. The RSD values for cefmetazole (n = 9) and cefoxitin (n = 9) are given in Table 2. The LOD in urine was determined to be 4 μg ml^{-1} for cefmetazole and 5 μg ml^{-1} for cefoxitin (corresponding to 3.2 and 4.1 ng on column). The LOQ in urine was determined to be 15 μg ml^{-1} for both cefmetazole and for cefoxitin. This was the lowest standard analysed and represented the lower limit of linearity.

Control recovery

Recovery for cefmetazole spiked serum controls was >97% (n = 16) and for cefoxitin spiked controls was >95% (n = 16; Table 3). Recovery from spiked urine controls was >97% for both cefmetazole (n = 16) and cefoxitin (n = 9; Table 4).

Discussion

The removal of probenecid and the quantitation of cefmetazole and cefoxitin in clinical serum and urine specimens has been demonstrated with this column-switching technique (Figs 3 and 4). Figure 3 illustrates the serum concentration–time profiles for single IV doses of 2 g of either cefmetazole sodium or cefoxitin, in a volunteer both with

Table 3
Recovery of cefmetazole and cefoxitin from spiked serum

Concentration ($\mu g\ ml^{-1}$) Added	Found	SD	Recovery* (%)	RSD (%)
Cefmetazole (n = 16)				
4.86	4.83	0.406	99.3	8.4
24.3	24.4	1.03	100.2	4.2
122	119	3.37	97.6	2.8
Cefoxitin (n = 16)				
4.79	4.62	0.179	96.4	3.9
24.0	23.1	0.544	96.4	2.4
120	115	3.26	95.9	2.8

*Calculated by dividing the concentration found by the concentration added.

Table 4
Recovery of cefmetazole and cefoxitin from spiked urine

Concentration ($\mu g\ ml^{-1}$) Added	Found	SD	Recovery* (%)	RSD (%)
Cefmetazole (n = 16)				
60.0	61.8	1.4	103	2.3
240	243	5.6	101	2.3
600	586	14.5	97.7	2.5
Cefoxitin (n = 9)				
60.5	63.6	2.4	105	3.8
242	251	9.5	104	3.8
605	591	16	97.6	2.7

*Calculated by dividing the concentration found by the concentration added.

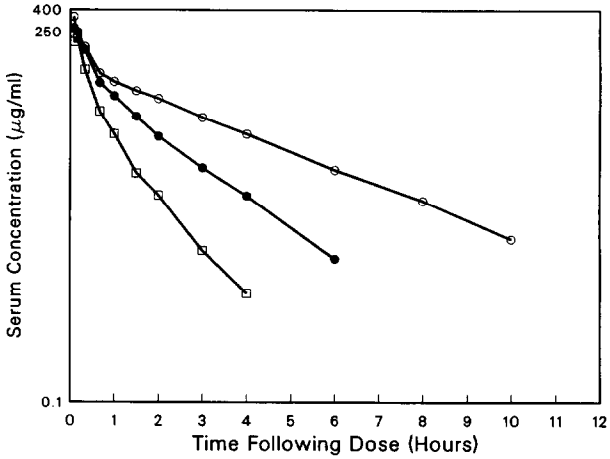

Figure 3
Serum concentration–time profile of a subject treated with a single 2-g dose of either cefmetazole sodium or cefoxitin. ○, Cefmetazole with preadministered probenecid; ●, cefmetazole without probenecid preadministration; □, cefoxitin without probenecid preadministration.

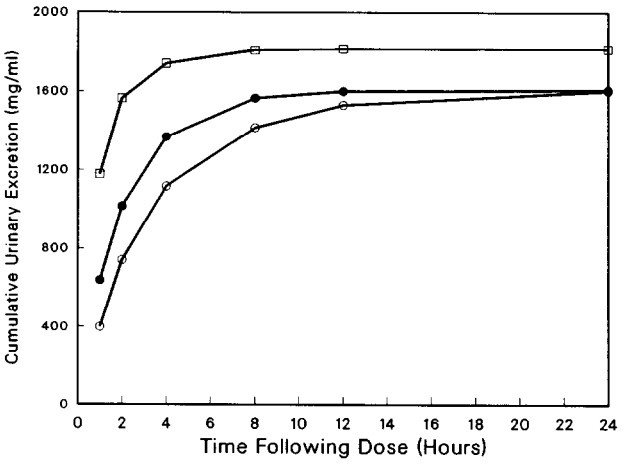

Figure 4
Cumulative urinary excretion profile of a subject treated with a single 2-g dose of either cefmetazole sodium or cefoxitin. ○, Cefmetazole with preadministered probenecid; ●, cefmetazole without probenecid administration; □, cefoxitin without probenecid preadministration.

and without preadministered probenecid. In this clinical study the effect of probenecid pretreatment was examined only with respect to cefmetazole. Figure 4 is a cumulative concentration–time profile in urine for the same volunteer and the same dosage regimen. These figures illustrate that adequate LOQs were achieved with this method to perform relevant pharmacokinetic calculations.

The method should be applicable to studies on other cephalosporin antibiotics. As shown in the above data, cefmetazole and cefoxitin displayed excellent linearity and precision. The method has also been used in the analysis of moxalactam and cefoperazone. Because of the similar polarity exhibited by these antibiotics, they elute in a narrow retention window, so the method is not appropriate for forensic analyses. However, in studies involving the treatment of subjects with one, known agent, this procedure has the necessary sensitivity and precision for pharmacokinetic calculations.

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